Johannes J. Geuze *et al.*
Serial No. 09/011,167

REMARKS

The Claimed Invention

The claimed invention is directed to an antigen presenting vesicle free from its natural surroundings.

The Pending Claims

Prior to entry of the above amendments, Claims 2-4, 6 and 13-17 are pending.

The Office Action

Claims 2-4, and 6, are objected to under 37 C.F.R. 1.75(c), as being of improper dependent form.

Claim 6 stands rejected under 35 U.S.C. 112 second paragraph, as being indefinite.

Claims 13, 2, 3, 4, and 6, and newly added claims 14-17 stand rejected under 35 U.S.C. 112 first paragraph, as not enabled by the specification.

Claims 13, 2, 3, 4, and 6, and newly added claims 14-17 stand rejected under 35 U.S.C. 112 first paragraph, as not meeting the written description requirement.

Response to the objections and rejections

In the response that follows, the Examiner's individual objections and rejections are provided in full text, as identified by indented small bold print, followed by Applicants response.

37 C.F.R. §1.75(c) Objection

Claims 2-4 and 6 are objected to under 37 C.F.R. §1.75(c), as being of improper dependant form for failing to further limit the subject matter of a previous claim. Applicant is required to cancel the claim(s), or amend the claim(s) to place the claim(s) in proper dependant form, or rewrite the claim(s) in independent form.

This rejection has been avoided by cancellation of Claims 2-4 and 6 without prejudice to renewal.

2

35 U.S.C. §112 Second Paragraph Rejection

Claim 6 is rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 6 recites the limitation "wherein said antigen presenting cell" in line 2. There is insufficient antecedent basis for this limitation in the base claim 13.

This rejection has been avoided by cancellation of Claim 6.

35 U.S.C. §112 First Paragraph Rejection (enablement)

Claims 13, 2, 3, 4, and 6, and newly added claims 14-17, are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for an antigen presenting vesicle comprising a membrane and a MHC class II protein wherein said antigen presenting vesicle is obtainable from an antigen presenting cell, does not reasonably provide enablement for an antigen presenting vesicle comprising a membrane and any MHC Class I protein, or any functional derivative or fragment thereof, wherein said antigen presenting vesicle is obtainable from any cell. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

With regard to claims 2-4 and 6, Applicant has stated that these claims have been deleted, though as discussed supra, there is no record of this. So until said claims are cancelled, the instant rejection of said claims is maintained.

Claims 2-4 and 6 have now been cancelled.

With regard to newly amended claim 13 and newly added dependant claims 14-17, the rejection has been maintained in part. Applicant contends that the rejection has been avoided in part by amending claim 13, so that claims 13-17 recite a cell that is a B lymphocyte, and by deleting the phrase "or a functional derivative or fragment thereof". The examiner agrees that said amendments have removed part of the instant rejection with regard to enablement of any antigen presenting cell and with regard to any functional derivative or fragment of a MHC Class I protein.

However, the instant specification is still not enabling regarding how to make and use an antigen presenting vesicle obtainable from a B lymphocyte comprising a MHC Class I protein as recited in newly amended claim 13 and newly added claims 14-17. Applicant notes that the examiner has noted that the instant specification discloses in its examples an antigen presenting vesicle derived from B cells. The examiner further notes presently that the instant specification does not disclose that said antigen presenting vesicle derived from B cells actually comprises MHC Class I proteins, but only discloses that said vesicles comprise MHC Class II proteins. Applicant contends that lymphocytes such as a human B cell line used to generate vesicles in the present invention, generally express MHC Class I protein, with which the examiner agrees. Applicant further contends that said Class I expression in B cells, in part explains the presence of Class I proteins on lymphocyte derived vesicles; however, the examiner can not find support for said contention in the instant specification. It is noted that Tse et al (Journal of Experimental Medicine (1984) 159(1):193-207 teaches that the vesicles showing MHC class I molecules were not found in B cells, see entire article, including the Abstract). Therefore, it would require undue experimentation to predict which antigen presenting vesicles derived from B cells comprise a MHC Class I protein without further guidance from the specification, or without objective evidence that the B cell derived vesicles exemplified in the instant specification comprise MHC Class I proteins.

Therefore, though Applicant's arguments have been fully considered, they are not persuasive, and the rejection is maintained in part, essentially for the reasons of record.

This rejection is respectfully traversed because the general technical teaching of the specification is that the claimed vesicles contain Class I MHC molecules.

The present invention and the Zitvogel reference cited by the Examiner provide evidence that MHC class I and/or II molecules are present in vesicles derived from antigen presenting cells.

To prepare the claimed vesicles, all that is required as a starting material are cells expressing MHC class I and/or class II proteins and the methodology disclosed in the subject application. The Zitvogel reference confirms that culture supernatants of antigen-presenting cells, such as dendritic cells cited by Zitvogel (page 595, column 1, lines 5-21) or B lymphocytes as claimed (page 1, line 17; page 5, line 18, page 6, line 2), can be used to isolate vesicles comprising MHC class I and class II by differential ultracentrifugation of culture supernatants (Zitvogel, page 595, column 1, lines 5-21; and the present application, at page 2, line 25 through page 3, line 1). The vesicles isolated by Zitvogel were 60 – 90 nm in diameter (page 595, column 1, line 1) and in the present invention 60 – 80 nm (page 9, line 7).

Although the claimed antigen-presenting vesicles are exemplified by identifying the presence of MHC class II molecules, the specification teaches the presence of MHC class I molecules on lymphocyte multivesicular MIICs (intracellular compartments bearing MHC class II molecules). The post-filing publication of Zitvogel, *et al.* confirms the operability of the method for obtaining the claimed antigen-presenting vesicles, and that multivesicular MIICs can comprise MHC I and/or MHC I molecules. The Zitvogel publication shows that differential centrifugation and fractionation over a linear sucrose gradient, *which was first disclosed in the instant specification*, may be used to isolate exosomes that comprise MHC I and/or MHC II molecules, and thus confirms that undue experimentation is not required. Western blot and immunoprecipitation analyses to detect the presence of MHC I molecules as well as MHC II molecules can be carried out using techniques well known to those in the art at the time of filing of the application, and by following the procedures taught in the

instant specification (page 3, line 1 through page 4, line 11; page 8, line 4 through page 10, line 9; and page 12, line 31 through page 13, line 13 of the original specification), but using anti-MHC class I antibodies readily available to those in the art (*see* Zitvogel *et al.*, cited by the Examiner, as well as Scott et al. (1995) *J. Immunol.* 155:143-148; Kaufman, *et al* (1995) *Proc. Natl. Acad. Sci.* 92:6484-6488; Atta, *et al* (1995) *Clin. Exp. Immunol.* 101:121-126; Khilko, *et al* (1995) *J. Immunol. Methods* 183:77-94; Rangel, *et al* (1995) *Eur. Cytokine. Net* 6:195-202, cited by Applicants on April 17, 2001 in their response to the Final Rejection). Therefore, by following the detailed exemplification provided in the specification for obtaining vesicles containing MHC Class II molecules, the claimed vesicles can be obtained without undue experimentation. One of skill in the art is not, as suggested by the Examiner, reduced to predicting which antigen presenting vesicles derived from B cells comprise MHC Class I protein, rather the person of skill can detect the protein by any of a variety of techniques well known in the art including the use of readily available anti-MHC class I antibodies.

The Tse reference is not a contemporary reference and does not support the Examiner's position.

The Tse et al reference cited by the Examiner in support of her position was published in 1984, some eleven years prior to the priority date of the subject application. Applicants' are able to find only a single sentence in the entire publication (page 204, lines 10-12) relating to B cells and internalization of MHC class I molecules. There are no data nor are there any citations to references containing data, and therefore no way for one of skill in the art to evaluate the accuracy of the statement. The reference is simply not enabling for that which it is cited. Furthermore, the sentence says only that Class I MHC molecules are excluded from the vesicles that are internalized that contain Class II MHC molecules; it does not say that B cells do not internalize Class I MHC molecules in vesicles, the proposition for which

5

Johannes J. Geuze *et al.*
Serial No. 09/011,167

the Examiner is citing the reference.

Zitvogel et al (1998) reports that MHC Class I molecules were observed in vesicular structures in B cells contrary to the teaching ascribed to Tse et al .

Zitvogel et al (already of record), published after the priority date of the subject application supports the teaching of the specification and provides contrary teaching to that ascribed by the Examiner to Tse et al. On page 598, second column, first full paragraph, Zitvogel et al report that Class I molecules are located on vesicular structures in B cells. Furthermore, treatment of the cells with cycloheximide was used to show that the Class I molecules in the vesicular structures were derived from the plasma membrane. Attached is a recent publication by one of the inventors (Wubbolts et al (2003)) which discloses "the abundant presence of MHC class I and class II" in human B-cell derived membrane vesicles (see abstract). The technique used for isolation of vesicles is essentially the same as that of the subject application. The Examiner is respectfully requested to withdraw this rejection.

35 U.S.C. §112 First Paragraph Rejection (written description)

Claims 13, 2, 3, 4 and 6, and newly added claims 14-17, are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention.

Response to Arguments

Applicant traverses the rejection on the grounds that language of the claims was present in the PCT application from which the instant application follows. However Applicant does not address the aspect of the instant rejection which notes tha the instant specification foes not describe the antigen presenting vesicle obtainable from a B cell that comprises a MHC Class I protein. It is noted that the instant specification describes an antigen presenting vesicle obtainable from a B cell wherein said vesicle comprises a MHC Class II protein, but does not describe that said vesicle (or any vesicle) comprises MHC Class I protein. Therefore, though the specification describes an antigen presenting vesicle obtainable from a B cell, wherein said vesicle comprises a MHC Class II protein, one of skill would not know they were in possession of an antigen presenting vesicle obtainable from a B cell, wherein said vesicle comprises a MHC Class I protein, without further description from the instant specification.

Therefore, though Applicant's arguments have been fully considered, they are not persuasive, and the rejection is maintained, essentially for the reasons of record.

6

Claims 2-4 and 6 have been cancelled. As to the remaining claims, this rejection is respectfully traversed because the Examiner has failed to explain why a person skilled in the art would not recognize that the inventor had possession of the claimed subject matter at the time the application was filed; a prima facie case of unpatentability has not been made.

Applicants have taught that lymphocytes such as the human B cell line used to generate vesicles in the present invention generally express MHC Class I protein therefore vesicles derived from such lymphocytes inherently contain Class I proteins.

A claim need not be described literally or "in haec verba" in order for the specification to satisfy the written description requirement. A structure, process or property not explicitly described may satisfy the requirement if the structure, process, or property is inherent in what is described.

Applicants have described generally in the specification that vesicles containing MHC Class I and/or Class II molecules can be obtained and have described that antigen presenting cells such as B cells can be used as a source of such vesicles. See for example page 6, lines 1-6 of the instant specification. It was known in the art, prior to the priority date of the subject application that B lymphocytes such as the human B cell line used to generate vesicles in the present invention express Class I MHC molecules. The specification describes that B lymphocytes secrete membrane vesicles that express MHC molecules. Applicants also described that MHC molecules were internalized in vesicles that could be isolated. It therefore follows that vesicles obtained starting with a source of cells that express MHC Class I protein on their surface inherently will contain MHC class I protein. The subject matter of the claims appears in the claims originally filed in the PCT application from which the subject application entered national phase in the US. Thus the applicants have conveyed with reasonable clarity to those skilled in the art that as of their priority date they were in possession of the claimed invention and the Examiner is respectfully requested to withdraw this
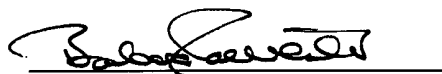
Johannes J. Geuze *et al.*
Serial No. 09/011,167

rejection.

CONCLUSION

In view of the above amendment and remarks, it is submitted that this application is now ready for allowance. Early notice to that effect is solicited. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned at (831) 648-3090.
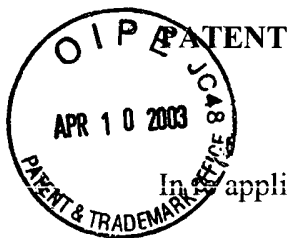
Respectfully submitted,

Date: _April 3, 2003_

Barbara Rae-Venter

Barbara Rae-Venter, Ph.D.
Reg. No. 32,750

Rae-Venter Law Group, P.C.
PO Box 1898
Monterey, CA 93942-1898
Phone: (831) 648-3090
Facsimile: (831) 242-0137

BRV/mnb

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: **Johannes J. Geuze**, *et al.*

Serial No.: 09/011,167

Filed: **October 5, 1998**

For: **CELL DERIVED ANTIGEN PRESENTING VESICLES**

Examiner: DeCloux, Amy

Art Unit: 1644

MARKED UP VERSION OF CLAIMS

Box After Final
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

The Examiner is respectfully requested to make the following amendments. A clean copy of the text of the claims following the amendments is attached hereto

IN THE CLAIMS:

Cancel Claims 2-4 and 6.

13.   (Reiterated) An antigen presenting vesicle free from its natural surroundings, said vesicle comprising:

a membrane, a major histocompatibility complex (MHC) Class I protein and one or more at least partially processed antigens bound to said MHC class I protein and wherein said antigen presenting vesicle is obtainable from a B lymphocyte.

14.   (Reiterated) The antigen presenting vesicle according to Claim 13, wherein said vesicle is obtained by the step of recovering a 70,000 x g pellet obtained by differential centrifugation of membrane-containing fractions of cell culture media or lysates of B lymphocytes containing said MHC Class I protein.

15.   (Reiterated) An antigen presenting vesicle free from its natural surroundings, said vesicle comprising:

a membrane, a major histocompatibility complex (MHC) Class I protein and one or more at least partially processed antigens bound to said MHC Class I protein, and wherein said antigen presenting vesicle is derived from culture media of B-lymphocytes containing said MHC Class I protein.

16.   (Reiterated) An antigen presenting vesicle free from its natural surroundings, said vesicle comprising:

a membrane, a major histocompatibility complex (MHC) Class I protein and one or more at least partially processed antigens bound to said MHC Class I protein, wherein said antigen presenting vesicle is obtained by the step of recovering a 70,000 x g pellet obtained by differential centrifugation of membrane-containing fractions of cell culture media or lysates of B-lymphocytes containing said MHC Class I protein.

17.   (Reiterated) An antigen presenting vesicle free from its natural surroundings, said vesicle comprising:
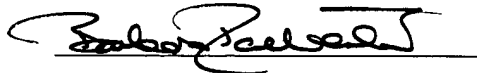
a membrane, a major histocompatibility complex (MHC) Class I and Class II proteins and one or more at least partially processed antigens bound to said MHC proteins, wherein said antigen presenting vesicle is obtained by the step of recovering a 70,000 x g pellet obtained by differential centrifugation of membrane-containing fractions of cell culture media or lysates of B-lymphocytes containing said MHC Class I and Class II proteins.

Respectfully submitted,

Date: _April 3, 2003_

[signature]

Barbara Rae-Venter, Ph.D.
Reg. No. 32,750

Rae-Venter Law Group, P.C.
PO Box 1898
Monterey, CA 93942-1898
Phone: (831) 648-3090
Facsimile: (831) 242-0137

BRV/mnb
Enclosures

Proteomic and biochemical analyses of human B cell-derived exosomes:

Potential implications for their function and multivesicular body formation

#R.W. Wubbolts, #R.S. Leckie, #P.T.M. Veenhuizen, °G. Schwarzmann, #W. Moebius. *J. Hoernschemeyer, #J.W. Slot, #H.J. Geuze, and #W. Stoorvogel§

#Department of Cell Biology, AZU G02.525, Heidelberglaan 100, Utrecht University Medical Centre and Institute of Biomembranes, 3585 CX Utrecht The Netherlands

°Kekule-Institut fuer Organische Chemie und Biochemie der Universitaet Bonn, Gerhard-Domagk-Str. 1, D-53121 Bonn, Germany

running title: Proteomic and biochemical analyses of B cell-derived exosomes

§Corresponding author:
W. Stoorvogel
University Medical Center Utrecht
Department of Cell Biology
AZU G02.525
Heidelberglaan 100
3584 CX Utrecht, The Netherlands
Phone: (31) 30.2507577 or -6551, fax: (31) 30.2541797
Email: W.Stoorvogel@Lab.AZU.NL

ABSTRACT

Exosomes are 60-100 nm membrane vesicles that are secreted into the extracellular milieu as a consequence of multivesicular body fusion with the plasma membrane. Here we determined the protein and lipid compositions of highly purified human B cell-derived exosomes. Mass spectrometric analysis indicated the abundant presence of MHC[e] class I and class II, Hsc70, Hsp90, integrin α4, CD45, moesin, tubulin (α and β), actin, Gᵢα2 and a multitude of other proteins. An α4-integrin may direct B cell-derived exosomes to follicular dendritic cells, which were previously described as potential target cells. Clathrin, Hsc70 and Hsp90 may be involved in protein sorting at multivesicular bodies. Exosomes were also enriched in cholesterol, sphingomyelin and ganglioside GM3, lipids that are typically enriched in detergent-resistant membranes. Most exosome-associated proteins, including MHC class II and tetraspanins, were insoluble in CHAPS-containing buffers. Multivesicular body-linked MHC class II was also resistant to CHAPS while plasma membrane-associated MHC class II was readily solubilised. Together, these data suggest that recruitment of membrane proteins from the MVB limiting membranes into internal vesicles may involve their incorporation into tetraspanin-containing detergent-resistant membranes.

INTRODUCTION

Maturing endosomes accumulate vesicles in their lumen, resulting in their transformation into multivesicular bodies (MVB) (1). These vesicles are formed by inward budding of the endosomal limiting membrane and contain a selected cargo. Proteins that are sorted to the internal vesicles of MVB potentially may have three distinct fates. The first possibility is exemplified by ligand-activated epidermal growth factor receptor, which is ultimately transferred to lysosomes for degradation (2). A second possibility is that proteins may be stored temporarily in MVB, as observed for MHC class II in immature dendritic cells (3). MHC class II carrying MVB in dendritic cells have also been termed MHC class II compartments (MIIC), in accordance with similar structures in B cells (4). MIIC play a crucial role in peptide loading of MHC class II. In pathogen-stimulated dendritic cells, the internal vesicles of MVB fuse back with their limiting membrane, thereby allowing subsequent transfer of peptide-loaded MHC class II to the plasma membrane (3). The third potential fate of vesicles within MVB is their release into the extracellular environment as a consequence of fusion of the MVB limiting membrane with the plasma membrane. These secreted MVB-derived vesicles have been called exosomes, which, depending on their source, may serve a multitude of functions (5-7).

Exosomes are released by a great number of cell types, including reticulocytes (5), cytotoxic T cells (8), B lymphocytes (9;10), dendritic cells (11-13), mast cells (14), platelets (15), and intestinal epithelial cells (16). The biological functions of exosomes are generally unclear. Increasing evidence, however, suggests that exosomes from haematopoietic cells may serve as intercellular communication vehicles that assist immune responses (6;7). For example, B cell-derived exosomes that carry peptide-loaded MHC class II were demonstrated to stimulate

CD4+ T cells (17) and to specifically bind follicular dendritic cells (18) in vitro. Furthermore, exosomes derived from cultured dendritic cells that were loaded in vitro with tumor-derived peptides on MHC class I stimulated cytotoxic T lymphocytes both in vitro and in vivo (11).

Functions of exosomes should be reflected by their protein composition. Given that exosomes are formed as the internal vesicles of MVB, exosomes can be expected to also contain factors required for MVB formation and protein sorting therein. Immuno-electron microscopic studies, Western blot analyses and peptide mass mapping of exosomes derived from dendritic cells (12;13), B lymphocytes (9;10), intestinal epithelial cells (16) and other cell types revealed the presence of common as well as cell type specific proteins. For example, MHC class II is especially enriched in exosomes derived from B-lymphocytes, dendritic cells, mast cells and intestinal epithelial cells. Ubiquitous proteins in exosomes include cytoplasmic proteins, such as tubulin, actin and actin-binding proteins, the heat shock proteins Hsc70 (also named Hsc71 or Hsp73) and Hsp90, and trimeric G proteins, as well as membrane proteins, such as members of the tetraspanin family (CD9, CD63, CD81, CD82). Sorting of a number of membrane proteins into the MVB pathway involves ubiquitination of their cytoplasmic domain (19) and binding of these acquired ubiquitin-moieties to Tsg101 (20). Indeed, Tsg101 (12) as well as c-Cbl (21), a ubiquitin ligase required for ubiquitination of activated epidermal growth factor receptor (2), have been detected in isolated exosomes. Alternatively, membrane proteins may rely on ubiquitinated adaptors for their sorting in MVB (20;22). Importantly, sorting of at least some proteins into the MVB pathway occurs independently of the ubiquitin system (23). The molecular factors and mechanism(s) behind such alternative sorting processes in MVB are unknown and the analysis of exosomes may help their discovery.

In an approach to understand more about the formation and function of exosomes we

developed a protocol that yielded highly purified exosomes from human B cells and studied

their molecular content and biochemical properties. Based on exosome characteristics, we

propose a model in which the incorporation of proteins into tetraspanin networks and DRM at

the MVB's limiting membrane may be conditional for their sorting into MVB's internal

vesicles.

MATERIALS AND METHODS

Antibodies, SDS-PAGE and Western blotting

Rabbit anti-DR (24) was kindly provided by Dr. H.L. Ploegh (Harvard Medical School, MA),

rabbit anti-MHC class II β-chain by Dr. N. Barois (INSERM-CNRS de Marseille-Luminy,

France) and the mouse monoclonals anti-CD81 (clone M38) and anti-CD82 (clone, C33) by

Dr. O.Yoshie (Kinki University School of Medicine, Osaka). Mouse monoclonal anti-MHC

class II (CR3/43) was from DAKO (Glostrup, Denmark), mouse monoclonal anti-CD86

(BU63) from Ancell (Lauflingen, Switzerland), mouse monoclonal anti-CD63 (435; CLB1/2)

from CLB (Amsterdam, The Netherlands), and polyclonal rabbit anti-biotin from Sanver Tech

(Boechout, Belgium). For the analysis of exosomes and cell fractions by SDS-PAGE and

Western blotting, samples were incubated for 15 min at 65°C in urea-containing sample

buffer (5% SDS, 9 M urea, 10 mM EDTA, 2.5% β-mercaptoethanol, 120 mM Tris-HCl pH

6.8). Proteins were separated on 12.5%, 10% or 7.5% polyacrylamide gels (SDS-PAGE). For

Western blotting, proteins were transferred from polyacrylamide gels to Immobilon-P

membrane (Millipore, Bedford, MA). The membranes were blocked an 1 probed with

antibodies in PBS containing 5% (w/v) non-fat dry milk Protivar (Nutricia, Zoetermeer, The

Netherlands) and 0.1% (w/v) Tween 20. Primary antibodies were probed with HRP-

conjugated secondary antibodies (DAKO, Glostrup, Denmark) and detected by enhanced

chemiluminescence (Roche, Basel, Switzerland).

Cell culture and exosome isolation

RN cells (HLA-DR15⁺) were cultured as described (9). We observed that FCS contains

exosomes (data not shown). To exclude bovine exosomes, cells were cultured in medium

supplemented with FCS that had been ultracentrifuged for 60 min at 14,000 x g_max. RN-

derived exosomes were routinely isolated from 800 ml culture media containing

approximately 10^8 RN cells. As a first isolation step, exosomes were collected from the media

by differential centrifugation, as described (9). In short, Cells were removed by centrifugation

for 10 min at 200 x g. Supernatants were collected and centrifuged sequentially twice for 10

min at 500 x g_{max}, once for 15 min at 2,000 x g_{max}, once for 30 min at 10,000 x g_{max} and once

for 60 min at 70,000 x g_{max} using a SW27 rotor (Beckman Instruments, Inc., Fullerton, CA).

Exosomes pelleted at the final centrifugation step and were resuspended in PBS and re-

pelleted at 70,000 x g_{max}. The final pellet routinely contained ~100 µg of protein. Re-pelleted

exosomes were resuspended in 5 ml 2.6 M sucrose, 20 mM Tris/HCl, pH 7.2 and floated into

an overlaid linear sucrose gradient (2.0-0.25 M sucrose, 20 mM Tris/HCl, pH 7.2) in a SW41

tube for 16 hours at 270,000 x g_{max}. Gradient fractions of 1 ml were collected from the bottom

of the tube and analysed for the presence of MHC class II and tetraspanins by Western

blotting. When indicated, gradient fractions were diluted with 3 ml PBS each, centrifuged for

60 min at 350,000 x g_{max}, and the pellets analysed by SDS-PAGE and Coomassie blue

staining. As a final purification step, 750 µl samples of pooled exosome-containing gradient

fractions were added to 200 µl Dynabeads M-450 (~8x10^7 beads) coated with monoclonal

mouse anti-human MHC class II (Dynal Biotech, Oslo, Norway). As a negative control,

Dynabeads M-450 coated with goat anti-mouse IgG (Dynal) were used. The Dynabeads that

were added to the exosomes suspensions were first extensively washed with and resuspended

in PBS supplemented with 3 mg/ml BSA. For adsorption, samples were rotated end-over-end

for 16 h at 4°C. The beads were collected and washed once with PBS with the aid of a magnet

(Dynal). Non-adsorbed membranes were diluted with PBS and collected by centrifugation for

30 min at 200,000 x g_{max} in a SW50 tube.

Mass spectrometric protein analyses

Proteins from Dynabead-associated exosomes were segregated by SDS-PAGE, stained with

Coomassie blue, excised from the gels and analysed by mass spectrometry by Protana

(Denmark). In-gel tryptic digestion of proteins was performed as described (25).

Approximately 2 percent of the tryptic digest was analysed on a Bruker Reflex MALDI-ToF

mass spectrometer (Bruker, Bremen, Germany) and the obtained peptide maps were queried

against a non-redundant sequence databases. Search criteria; mass accuracy: 50 ppm, tryptic

peptides, allowed missed cleavage sites: 1. Samples not unambiguously identified by peptide

mass finger prints were purified and concentrated using "home built" Perros R2 (Applied

Biosystems, California, USA) micro columns before sequence analysis. The tryptic peptides

were sequenced on a QSTAR quadrupole-ToF mass spectrometer (Sciex, Ontario, Canada)

equipped with a nanoelectrospray source (ProtanaEngineering, Denmark). Prior to analysis,

the mass spectrometer was calibrated to a mass accuracy of 20 ppm and a resolution of 9500.

The data were processed with PPSS2 (Protana's Proteomics Software Suite) and the peptide

sequence tags obtained were queried against a non-redundant sequence databases (26). Search

criteria; MS mass accuracy: 1.1 Da, MSMS accuracy: 0.1 Da, tryptic peptides, allowed

missed cleavage sites: 1. For verification of a retrieved peptide sequences theoretical patterns

were compared to the obtained CID spectra.

Lipid analysis

PBS washed RN cells, Dynabead-associated exosomes and ultracentrifuged non-adsorbed

membranes were suspended in a total volume of 3 ml of chloroform/methanol (1/1, v/v) and

lipids were extracted overnight at 40 °C. The suspensions were then centrifuged for 10 min at

2000 x g and the clear supernatants were dried in a stream of nitrogen. The residue was

dissolved by first adding 60 µl chloroform followed by 0.96 ml methanol and 0.94 ml water.

Each resulting solution was freed of salts and sucrose by reverse phase chromatography. For this procedure small pieces of silanised glass fiber wadding were introduced into glass Pasteur pipettes and a suspension (1.5 ml, 1 to 3 v/v in methanol) of LiChroprep RP18 (40-63 µm, Merck, Darmstadt, Germany) was added. These columns were subsequently washed 3 times with 1 ml chloroform/methanol (1/1, v/v), 3 x 1 ml methanol and 3 x 1 ml water. Then the sample solution was applied and the column was washed with 3 x 1 ml water. Bound lipids we[...]ted with 3 x 1 ml methanol and 3 x 1 ml chloroform/methanol (1/1, v/v). The eluate was dried under a stream of nitrogen, dissolved in 0.10 ml chloroform/methanol (1/1, v/v), and analyzed by thin-layer chromatography (TLC). TLC was performed in a horizontal development chamber (Camag, Muttenz, Switzerland) using 10 cm x 10 cm silica gel-coated thin-layer plates (Merck, Darmstadt, Germany) and a mixture of chloroform/methanol/water (65/25/4, by vol.) as the mobile phase. Lipids were visualized by fine-spraying the developed plates with copper sulfate (10% w/v) in phosphoric acid (8% w/v) followed by charring for 8 min at 180 °C. For quantitation the charred plates were scanned and the data analyzed by phosphoimaging using the TINA software version 2.0 (Raytest, Germany) and compared with known amounts of reference lipids (cholesterol, dioleoyl phosphatidyl ethanolamine, bovine heart cardiolipin, dioleoyl phosphatidyl choline and sphingomyelin containing stearic or ne[...]c acid). For the distinction between cholesterol and its esters thin-layer plates were developed in n-hexane/diethyl ether/acetic acid (60/40/1, by vol.). For an unambiguous identification extracted lipids from B-lymphocytes, which were grown in quantity in the presence of $[1-^{14}C]$ sodium acetate (Amersham, England), were separated into uncharged and acidic lipids prior to preparative TLC Lipids were traced by their radioactivity, scraped and extracted with chloroform/methanol (1/1, v/v). The lipid extracts were then subjected to mass spectrometry. The separation according to charge was by DEAE-Sephadex A 25 chromatography in chloroform/methanol/water (3/7/1, by vol). Acidic lipids were eluted with

chloroform/methanol/1M ammonium acetate (3/7/1, by vol) and freed of salts, short chain fatty acids and other less hydrophobic material by reverse phase chromatography on LiChroprep RP18 as described above.

For electrospray-MS (ESI-TOF-MS), mass spectra were recorded in the negative or positive ion mode for acidic or uncharged lipids, respectively, on a Q-TOF 2 mass spectrometer (Micromass, Manchester, UK) equipped with a nanospray source. Lipids were dissolved in chloroform/methanol 1:1 (v/v). Solutions were injected into the mass spectrometer by glass capillaries (long type; Protana, Odense, Denmark) using a capillary voltage of 1000 V and a cone voltage of 50 V at 70°C. Instrument calibration was done with a mixture of sodium iodide and cesium iodide in 50 % aqueous acetonitrile with 0.1 % formi.: acid. For MS/MS experiments argon was used as collision gas and fragmentation was observed at energy values from 20 – 50 eV.

For matrix-assisted laser desorption/ionization time-of-flight-MS (MALDI-TOF-MS), measurements were done on a TOFSpec E (Micromass, Manchester, UI:) in positive or negative ion mode with an accelerating voltage of 20 kV. For lipid samples matrix solutions of 2,5-dihydroxybenzoic acid were used in a concentration of 10 mg/ml in methanol. Spectra were externally calibrated by using suitable reference substances.

Density gradient electrophoresis and cell surface biotinylation.

RN cells were collected and washed with PBS by centrifugation at 4°C for 10 min at 200 x g.

The cell surface was biotinylated for 15 min at 0°C using 5 mg/ml sulfo-NHS-biotin (Pierce, Rockford, IL). Excess sulfo-NHS-biotin was quenched with 10 mM NH_4Cl in PBS for 20 minutes at 0°C after which the cells were collected and washed twice with homogenisation buffer (10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, 0.25 M sucrose, pH 7.4) by centrifugation. Cells were homogenised using an EMBL-cell cracker (ball 8.011 applying 10

strokes) and nuclei were removed by centrifugation at 900 x g_{max} for 3 minutes. The post-nuclear supernatant was treated with trypsin (25 µg/mg protein) for 15 minutes at 37°C after which trypsin inhibitor (100 µg/ mg protein; Sigma, St Louis, MO) and protease inhibitor mix (Roche, Basel, Switzerland) were added. Density gradient electrophoresis was performed as described (27) for 30 minutes at a constant current of 10 mA. Fractions of 500 µl were collected from the anodic side and analysed by Western blotting for MHC class II β-chain.

P   membrane proteins were detected by probing Western blots with streptavidin-peroxidase (Sigma, St Louis, MO) and enhanced chemiluminescence. β-hexosaminidase was determined as described (28) and total protein was assayed using the Biorad protein assay (Biorad, München, Germany).

To compare plasma membrane-associated MHC class II with exosomes-associated MHC class II, intact cells and isolated floated exosomes were biotinylated at 0°C as described above. After detergent extraction and ultra-centrifugation, pellets were suspended in 0.5 ml PBS containing 1% TX100, 10 mg/ml BSA, 2 mM EDTA, 0.2 mM PMSF and 30 µl Neutravidin-sepharose beads (Pierce, Rockford, IL) for 16 hrs. The beads were washed with PBS and analysed for bound MHC class II by Western blotting.

Solubility assay and floatation

Gradient fractions (3 ml in total) containing floated exosomes were diluted with 26 ml PBS. Aliquots of 3.6 ml were mixed with 0.4 ml PBS containing 10% CHAPS, 10% TX100, 10 mM EDTA, 10 mM MgCl$_2$ and/or 100 mM methyl-β-cyclodextrin (Sigma, St Louis, MO) as indicated. The samples were then centrifuged in SW60 tubes for 1 hr at 350,000 x g_{max}. Pellets were analysed by SDS-PAGE followed by Coomassie blue staining or Western blotting.

11

To demonstrate association of low-density lipid material with MHC class II after detergent solubilisation we performed floatation assays. Floated exosomes from sucrose density gradients were pelleted as above and suspended in 500 µl 2.5 M sucrose, 0.5 mM CaCl$_2$, 0.5 mM MgCl$_2$, 1 mM PMSF, 20 mM Hepes/NaOH pH 7.2 in the presence or absence of 1% CHAPS by 10 passages through a 23G needle mounted on a syringe. The suspension was overlaid with a 2 M – 0.4 M sucrose gradient in 20 mM Hepes/NaOH pH 7.2 and centrifuged for 4 hours at 200,000 x g_{max}. Fractions were collected from the bottom of the tube and analysed by SDS-PAGE followed by Coomassie blue staining or Western blotting.

Immunoelectron microscopy

Perfringolysin O modified with subtilisin Carlsberg and subsequent biotinylation was kindly obtained from Dr. Ohno-Iwashita (29). Cells were prepared for cholesterol labelling as detailed elsewhere (30). Perfringolysin O-labelled sections were fixed in 1% glutaraldehyde and labelled with rabbit polyclonal anti-DR (24) or anti CD63 followed by protein A coupled to 15 nm gold. After another fixation with 1% glutaraldehyde, sections were incubated with anti-biotin antibodies (4 µg/ml) followed by incubation with protein A coupled to 10 nm gold. Sections were then examined and photographed at 80 kV with a JEOL 1200 EX electron microscope (Tokyo, Japan).

12

RESULTS

Purification of secreted human exosomes

Exosomes secreted by the human B-cell line RN were purified in three sequential steps. In the first step we performed differential centrifugation to collect membranes from the culture media that sedimented between 300,000 g_{max} x min and 4,200,000 g_{max} x min (10;12). In the second purification step pelleted exosomes were washed and floated into sucrose density gradients to remove non-membranous serum protein (complexes). Exosomes, as identified by the presence of MHC class II, floated up to a density of ~1.15 g/ml (Figure 1B), consistent with previous observations (9;10). The gradient fractions were diluted with PBS, ultra-centrifuged and the pellets were analysed by SDS-PAGE and Coomassie blue staining (Figure 1A). The sample buffer used for SDS-PAGE was supplemented with urea (see materials and methods sections) as we found that this greatly enhanced the separation of exosome-associated proteins by SDS-PAGE. A number of proteins, possibly originating from FCS in the culture medium, remained in the bottom fractions (indicated by the arrows) while the majority of proteins co-distributed with MHC class II in the gradient. In addition to MHC class II, these proteins include CD86, and the tetraspanins CD37, CD53, CD63, CD81 and CD82 (data not shown) as previously identified by Western blotting (10). As a third purification step, gradient fractions containing MHC class II (fraction 6-8) were pooled and exosomes were immuno-adsorbed onto anti-MHC class II-coupled magnetic beads.

Membranes that did not associate with the beads were collected from the bead supernatant by ultra-centrifugation. Bead-associated and non-associated proteins were analysed by SDS-PAGE and Coomassie blue staining (Figure 2). The anti-MHC class II antibody-conjugated beads recovered nearly all proteins while beads coated with negative-control antibodies did

not collect any of these proteins. This indicates that all detected proteins were physically linked to MHC class II carrying exosomes.

Protein composition of exosomes

To analyse the identity of exosome-associated proteins, discernable Coomassie blue stained bands in figure 2, lane 1 were excised from the gel and analysed by mass spectrometry. Identified proteins are indicated in Figure 2 and listed in Table 1. Only proteins with a minimum of two matching peptides are shown. HLA encoded proteins, including MHC class I heavy chain and several MHC class II subtypes, are dominantly present in exosomes. Other membrane proteins that were identified include Na$^+$/K$^+$-ATPase, the receptor tyrosine phosphatase CD45, integrin α-4, and the receptor-associated inhibitory signalling molecule Gα2 which is linked to the cytoplasmic face of membranes by a palmitoyl anchor. Other identified proteins can be grouped in heat shock proteins (Hsp90α and Hsc70), cytoskeletal proteins (α and β tubulin and actin), a member of the ERM family of cytoskeleton-associated proteins (moesin) and a set of enzymes involved in glycolysis (GAPDH, pyruvate kinase, alpha enolase and fructose-bisphosphate aldolase A). Clathrin heavy chain-1 (CHC-1) and elongation factor 1A were detected as well. Murine immunoglobulin heavy chain originated from the anti-MHC class antibody that was conjugated to the Dynal beads. Tetraspanins were not detected by mass spectrometry, possibly because of low abundance and/or their poor resolution characteristics by SDS-PAGE (10).

Lipid composition of B-lymphocytes and exosomes

Lipids extracted from [1-^{14}C] sodium acetate labelled lymphocytes were separated according to charge and relative mobility by TLC, stained and quantified (Figure 3, lane 2; table II). The nature of these lipids was identified in parallel experiments in which the position of ^{14}C-

labelled lipids was determined on film rather than by staining. These were then extracted from the TLC plates and analysed by electrospray-time-of-flight-mass spectrometry (ESI-TOF-MS) and matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry (MALDI-TOF-MS) (Table III). Among the acidic lipids were ganglioside GM3 with palmitoyl or nervonoyl residues. The majority of acidic lipids were, however, composed of phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidic acid (PA), bismonoacyl glycerophosphate (BMP) and cardiolipin (CL) with varying fatty acyl moieties. Their fatty acid composition was deduced from tandem mass spectra collected in the negative ion mode. The amount of BMP exceeded that of CL (see Figure 3 A). The uncharged lipids were comprised of sphingomyelin (SM), cholesterol (Chol), phosphatidyl choline, phosphatidyl ethanolamine and ether lipids with an ethanolamine phosphoryl head group. Like GM3, sphingomyelin contained either palmitoyl or nervonoyl residues, resulting in two distinct bands by TLC. SM containing C16:0 was not separated from ganglioside GM3 by TLC (lower band of the double band in Figure 3A). Dynabead-bound exosomes and membranes from exosome-containing sucrose gradient fractions that did not bind to anti-MHC class II coated Dynabeads were analysed for their lipid content and compared with total cell membranes (Figure 3, table II). Exosomes were enriched in cholesterol (42 mol% versus 20 mol% in total cell membranes) and sphingomyelin and ganglioside GM3 (23 mol% versus 13 mol% in total cell membranes) on the expense of the presence of phosphatidyl ethanolamine and its respective ether lipids as well as phosphatidyl choline, phosphatidyl inositol, phosphatidyl serine and phosphatidic acid. As expected, cardiolipin, a lipid that is predominantly found in mitochondria, was absent from exosomes. Bismonoacyl glycerophosphate (BMP), also referred to as lysobisphosphatidic acid (LBPA), could also not be detected in exosomes.

Electron microscopic detection of cholesterol on exosomes

To determine the morphological distribution of cholesterol, cryosections of RN cells were labelled for cholesterol with 10 nm colloidal gold using perfringolysin O and examined by electron microscopy (30). The sections were double labelled for either MHC class II (15 nm gold) or CD63 (15 nm gold). MHC class II was detected on the plasma membrane, in MIIC and on exosomes within MIIC - plasma membrane fusion profiles. Consistent with previous observations (10), CD63 was found predominantly on the internal membranes of MIIC and exosomes and not at the plasma membrane. Figure 4 shows fusion profiles of MIIC with the plasma membrane. Consistent with the lipid analyses, cholesterol was predominantly present on secreted exosomes and much less abundant on the MIIC-limiting membrane and plasma membrane. We conclude, based on the morphological and biochemical characterisation of exosomes, that exosomes are relatively enriched in cholesterol.

Exosomes are detergent resistant

The enrichment in sphingomyelin, GM3 and cholesterol is a characteristic of so-called detergent-resistant membranes (DRM) or raft domains (31). Such domains are unusually resistant to solubilisation by non-ionic detergents (32). To investigate whether exosomes display DRM-like properties we determined their solubility in the presence of 1% TX100 or 1% CHAPS (figure 5). Sucrose gradient fractions containing exosomes (as in Figure 1) were pooled and washed with PBS. Aliquots were incubated as indicated for 30 min at 0°C or 37°C in the presence or absence of 1% CHAPS, 1% TX100, 1 mM EDTA, 1 mM MgCl2, and/or 10 mM methyl-β-cyclodextrin, and then centrifuged. Pellets were analysed for total protein content by SDS-PAGE and Coomassie blue staining (Figure 5A) and for the presence of MHC class II by Western blotting (Figure 5B). Many exosomal proteins, but not all, were resistant to solubilisation by CHAPS, independently of divalent cations or temperature. The

solubility of some proteins slightly increased when cholesterol was chelated with methyl-β-cyclodextrin at 37°C, suggesting that cholesterol is important for exosomal DRM. The solubility of exosomes was higher in TX100 compared to CHAPS, but remained incomplete. The same observations were made for exosome-associated MHC class II. MHC class II was solubilised to a significant degree only in the concomitant presence of CHAPS and methyl-β-cyclodextrin at 37°C or in the presence of TX100. DRM are characterised by the stable association of both proteins and lipids and, consequently, have a relative low buoyant density in sucrose density gradients. To test whether DRM are associated with exosomes, we performed floatation experiments for CHAPS treated exosomes (Figure 6). Pelleted exosomes were resuspended in 2.5 M sucrose containing 1% CHAPS and overlaid with a sucrose density gradient. After ultra-centrifugation, gradient fractions were analysed for the presence of MHC class II and the tetraspanins CD81 and CD63. About one third of each of these markers floated up into the gradient, indicating their association with DRM.

The solubility of MHC class II depends on its location.

Exosomes are formed as the internal vesicles of MVB/MIIC. Since the majority of MHC class II in MIIC of RN cells localizes to these internal vesicles (9), we hypothesized that MHC class in MIIC, like exosomes, should be resistant to solubilisation in CHAPS. To test this idea, we isolated MIIC from surface biotinylated cells by density gradient electrophoresis (Figure 7), a technique that segregates MIIC from other cellular membranes (33). Each fraction was tested for the presence of total protein, β-hexosaminidase (a marker for MVB and lysosomes) and biotinylated proteins (positioning the plasma membranes), and Western blotted for MHC class II. As expected, most MHC class II was found in plasma membrane containing fractions (fractions 16-21), but a significant amount was associated with MIIC, as indicated by its co-migration with β-hexosaminidase (fractions 5-10).

17

To compare exosomes with MIIC for the solubility of MHC class II, samples of pooled MIIC-containing fractions from density electrophoresis gradients and pooled exosome-containing sucrose gradient fractions were diluted with excess PBS, containing either CHAPS or TX100 or lacking detergent. After 30 min at 0°C or 37°C, DRM were pelleted by ultra-centrifugation and analysed for the presence of MHC class II by Western blotting (Figure 8). Exosomes and MIIC were similar with respect to the solubility of MHC class II (Figure 8A), consistent with the notion that the majority of MHC class II in MIIC localises to internal vesicles and that these vesicles are released as exosomes upon exocytic fusion of MIIC with the plasma membrane.

To compare the detergent solubility of MHC class II at the plasma membrane with that of exosomes, intact cells and isolated exosomes were first biotinylated. This procedure allowed selective labelling of the exoplasmic domain of plasma membrane or exosome-associated MHC class II. After the addition of CHAPS, insoluble MHC class II was pelleted by centrifugation and solubilised in TX100-containing PBS. Biotinylated MHC class II was collected using Neutravidin-conjugated sepharose beads and analysed by Western blotting. The solubilisation of biotinylated MHC class II from exosomes in CHAPS was inefficient (Figure 8B), possibly even more so than total MHC class II from non-biotinylated exosomes (Figure 8A). In contrast to exosome-related and MIIC-derived MHC class II, however, plasma membrane-derived MHC class II was entirely solubilised by CHAPS (Figure 8B). These data indicate that MHC class II is associated with DRM in exosomes and MIIC but not at the plasma membrane. Possibly, the incorporation of MHC class II in DRM at the MIIC-limiting membrane plays a role in its sorting into the MVB internal vesicles.

18

DISCUSSION

We developed a method to purify B cell derived exosomes to homogeneity, thus allowing determination of their protein and lipid composition. In a previous study we already demonstrated the presence of MHC class I and class II on B cell-derived exosomes (9;10), here we show that they are amongst the most prominent proteins. MHC class I and class II have also been detected on exosomes from dendritic cells (11), intestinal epithelial cells (16) an... ...lls (21). MHC class I was also found to associate with tumor derived exosomes (34) and MHC class II with mast cell derived exosomes (14). Previously, we also demonstrated by immuno electron microscopy and Western blotting that the tetraspanins CD63, CD37, CD53, CD81 and CD82 are heavily enriched on B cell derived exosomes and on the internal vesicles of MVB (10). One of these tetraspanins, CD63, is in fact also known as lysosome-associated membrane protein 3 (Lamp 3). Tetraspanins have also been demonstrated on exosomes derived from dendritic cells (11;12), intestinal epithelial cells (16), T cells (21), and platelets (15). In the current study we failed to detect tetraspanins by the mass spectrometric analyses.

Despite their relative enrichment, the only tetraspanin previously detected in exosomes by mass spectrometric analyses is CD9 (12), most likely because tetraspanins cannot be recovered as discrete bands from acrylamide gels (10;13). Tetraspanins comprise a large gr... ubiquitously expressed 25-50 kD proteins that contain a number of conserved residues (35). Tetraspanins associate with each other as well as with many Ig superfamily proteins, proteoglycans, integrins, growth factor receptors and signalling enzymes to form large transmembrane protein networks. Such networks are involved in a variety of processes at the plasma membrane such as cell adhesion, cell motility and signalling (36). Many of the interactions within these networks are relatively stable in the presence of detergents. Moreover, detergent solubility assays demonstrated the association of detergent-resistant lipids with tetraspanin networks providing them with raft/DRM-like properties (37).

It has been well established that at least fractions of MHC class II and MHC class I localize to membrane microdomains together with tetraspanins and integrins as measured by co-immunoprecipitation from detergent lysates (37-40), flow cytometric energy transfer methods (41) and competition assays (42). In mild detergents tetraspanins remain associated with microdomains resembling lipid rafts/DRM whereas at relatively harsh conditions they can be solubilised as protein webs that remain stable independently of lipid microdomains (37). The association of MHC class II with lipid rafts (43) as well as with tetraspan microdomains distinct from lipid rafts (44) has been proposed to facilitate antigen presentation. Although several of the above mentioned studies indicated the presence of such microdomains at the plasma membrane, their subcellular distribution has not been investigated systematically. MHC class II may associate with distinct sets of tetraspanins depending on its subcellular location (45). For example, CD82 associates with MHC class II in MIIC (46). Here we demonstrate that MHC class II is in detergent resistant DRM/protein webs at MVB rather than at the plasma membrane.

We found that exosomes are enriched in cholesterol, sphingomyelin and GM3. These lipids are characteristically enriched in rafts/DRM. These features, together with the presence of tetraspanins and the stable association of lipids with CHAPS-solubilised exosomal protein webs, indicate that exosomes contain protein/lipid complexes that can be described as webs or DRM/rafts. These webs or DRM/rafts may contribute to protein sorting in MVB, but may also play a role in the generation of membrane buds and even in membrane fission (31;47). We did not observe detectable amounts of BMP by TLC on exosomes. This is seemingly inconsistent with other studies in which BMP was detected either immunocytochemically on exosomes (18) or biochemically in a subcellular fraction containing the internal membranes of MVB (48). Possibly, the amount and distribution of BMP is cell type dependent. Furthermore, we

observed by immuno-electron microscopy that in RN cells BMP is enriched on multilaminar lysosomes rather than on MVB (Moebius, non-published results). Certain viruses, such as human cytomegalovirus (49) and HIV in macrophages (50) assemble at MVB in a process resembling the formation of MVB internal vesicles. T cells, in contrast to macrophages, assemble HIV predominantly at the plasma membrane in a process that requires elements of the same molecular machinery involved in MVB biogenesis (51). Interestingly, the membrane of HIV-1 is, like exosomes, enriched in cholesterol and sphingomyelin (52) and lipid rafts have been implicated in HIV-1 assembly and release (53).

At the plasma membrane, glycosylphosphatidylinositol anchored proteins have the tendency to be incorporated into DRM/rafts. The fact that glycosylphosphatidylinositol anchored proteins are also enriched in reticulocytes exosomes (54;55) is consistent with the idea that exosomes derive from DRM at the MVB limiting membrane. Gα subunits of heterotrimeric G proteins undergo palmitoylation and/or myristoylation on their amino-terminal ends and as a consequence are also targeted to lipid rafts (56). Given our current finding that exosomes contain $G_i\alpha2$, it is possible that $G_i\alpha2$ is targeted to the internal vesicles of MVB due to its incorporation into lipid rafts/DRM at the MVB limiting membrane. The presence of $G_i\alpha2$ in exosomes has also been reported for dendritic cell derived exosomes (12). The association of $G_i\alpha2$ with exosomes may be related either to general MVB functions or specifically to sorting of G-protein-coupled receptors at MVB (57;58).

The presence of Hsc70 in B cell derived exosomes is consistent with its detection in exosomes from dendritic cells (12), tumors (34) and maturing reticulocytes (59;60). Maturing reticulocytes dispose of their transferrin receptors by incorporating them into exosomes and it has been proposed that a direct interaction between Hsc70 and the transferrin receptor

cytoplasmic domain is associated with its targeting to exosomes (59;60). In addition to such chaperone functions, Hsc70 is also acting in other processes involving protein folding and unfolding. For example, Hsc70 has been demonstrated to regulate the disassembly of clathrin coats (61). Clathrin is most often associated with outward budding of membranes into the cytoplasm. However, clathrin has also been detected in non-curved lattices on MVB (62),(22) including MIIC (63). Clathrin in these coats has been demonstrated to associate with Hrs, an adaptor protein that binds directly to clathrin and ubiquitinated membrane proteins and is involved in the sorting of such proteins into the internal vesicles of MVB (22) (see also below). This process may result in the incorporation of clathrin, which we also detected in exosomes, and Hsc70 into MVB internal vesicles. Hsc70 forms complexes with other chaperones, including Hsp90 (64), another heat shock protein that we detected in exosomes. Consistent with their presence in exosomes, these molecular chaperone complexes have been demonstrated to function in the translocation of cytoplasmic protein substrates into a subset of lysosome-like organelles (64). Although this process has been interpreted to reflect protein translocation across the lysosomal outer membrane, targeting to the internal vesicles of MVB cannot be excluded.

In addition to actin we detected moesin, an actin-binding of the ezrin-radixin-moesin (ERM) family in exosomes. Moesin has been demonstrated to play a role in de novo actin assembly on phagosomal membranes (65). ERM proteins may play a role in budding processes as they are incorporated into rhabdoviruses (66) and HIV (67;68).

As indicated above, ubiquitination of membrane proteins serves as a signal for their sorting in MVB. Sequential association of sorting complexes termed ESCRT 1-3 to their ubiquitin moiety is thought to select membrane proteins for sorting into the MVB pathway (19;69;70).

All components that are required to recruit proteins into the MVB pathway, including ubiquitin, ESCRT complexes and the clathrin coat, are released from assembled cargo prior to the actual packaging into inwardly budding vesicles at the MVB limiting membrane. Dissociation of the ESCRT complexes seems to be regulated by the AAA-ATPase SKD1/Vps4 and interference with this process results in aberrant sorting in MVB (20;71). It should be noted, however, that not all proteins require ubiquitination for their sorting into the MVB pathway (23). Such proteins may partition into the inwardly budding vesicles due to intrinsic properties and preference to partition into raft-like microdomains or associate with tetraspanins. Endocytosed proteins that normally recycle, like the transferrin receptor and acetylcholinesterase, are, when aggregated by antibodies, mistargeted into the MVB pathway and subsequently secreted in association with exosomes (72). The hypothesis that protein clustering is an important determinant for entry into the MVB pathway is also supported by the observation that interference with transferrin receptor binding to Hsc70 increases its aggregation and association with exosomes (60). Similarly, MHC class II (73) and associated invariant chain (74) have been shown to bind directly to Hsc70, and this interaction may be important for the trafficking of MHC class II in MVB.

The transmembrane protein tyrosine phosphatase CD45 modulates the signal that is transduced via the B cell antigen receptor by regulating the phosphorylation state of Src family kinases and is required for normal B cell development, tolerance induction, and responsiveness to antigen (75). Interestingly, CD45 was found to be absent on T cell derived exosomes (21). The relevance of the specific association of CD45 with B cell derived exosomes is unclear. Na^+, K^+-ATPase is generally present at the plasma membrane. However, its cell surface expression can be regulated by endocytosis and it plays a regulatory role in the acidification of endosomes and lysosomes (76).

The presence of an α4 integrin on B cell-derived exosomes is intriguing. B cell selection involves their homing to follicular dendritic cells in the germinal centre in a process that is dependent on the interaction of α4 β1 with VCAM-1 (77). B cell (RN) derived exosomes bind in vitro to follicular dendritic cells and not to other cell types, suggesting that follicular dendritic cells are physiological targets for B cell derived exosomes (18). As for B cells, binding of exosomes to follicular dendritic cells may require integrin α4. In the germinal centre B cells recognize native antigens that are held in immune complexes at the surface of follicular dendritic cells by a set of different complement receptors. Binding of B cells to these antigens is an essential selection step for their differentiation into memory B cells. A prerequisite for the stimulation of T helper cells by follicular dendritic cells is their interaction with MHC class II. However, follicular dendritic cells do not synthesize MHC class II molecules themselves, but rather passively acquire peptide-loaded MHC class II (78), possibly by binding B cell derived exosomes through the interaction of α4 β1 with VCAM-1.

Table I. Proteins from figure 2 as identified by mass spectrometry.

Name	Accession code	Mass (Da)	Matched peptides	Coverage (aa) total / match
MHC class I heavy chain (MHC class I HC)	Q29638	40419	8	146/362 (40%)
MHC class II α-chain (HLA DRA)	V00523	19748	4	69-81/171 (40-47%)
MHC class II β-chain (HLA DR2)	Q29787	27065	3	72/237 (30%)
MHC class II β-chain (HLA DR2.2)	P01911	22964	4*	97/198 (49%)
MHC class II β-chain (HLA DQB1*0602)	L34105	26335	4	164/229 (72%)
T200 leukocyte common antigen (CD45)	Q16614	132381	7*	114/1143 (10%)
integrin α-4 (VLA 4)	P13612	116760	2*	16/1038 (2%)
$G_i\alpha2$	P04899	40890	8*	164/354 (46%)
heat shock protein 90-α (Hsp90 or Hsp86)	P07900	84542	1)	175/731(24%)
heat shock cognate 71 (Hsc70 or Hsp73)	P11142	70898	11	242/646 (37%)
clathrin heavy chain-1 (CHC-1)	Q00610	193384	4*	41/1675 (2%)
α-tubulin 1	P05209	50151	5	146/451 (32%)
β-tubulin 1	P07437	49759	6	119/444 (27%)
actin	P02577	41601	5	94/375(25%)
moesin	P26038	67689	14	154/576 (27%)
pyruvate kinase	P26038	57782	10	164/530 (31%)
α-enolase	P06733	47380	6*	78/433(18%)
glyceroaldehyde-3-phosphate dehydrogenase (GAPDH)	J04038	35922	4	69/335 (21%)
fructose-bisphosphate aldolase A (F-BPA-A)	P04075	39745	4*	63/363 (17%)
Na^+/K^+-ATPase α-1	P05023	1114208	12	217/1023 (21%)
elongation factor 1-α 1 (EF 1A)	X16869	50469	2*	29/462(6%)

Coomassie blue stained bands in figure 2, lane 1 were excised from the gel, trypsinised and

analysed by mass spectrometry for peptide mass finger prints. When mass finger prints did

not unambiguously match a specific protein, the peptides were sequenced by mass

spectrometry (indicated with *).

Table II. Relative lipid compositions as determined from Figure 3.

lipid	RN cells	exosomes	non-bound
cholesterol	19.6	42.1	55.5
PE + ether lipids	27.5	14.6	n.d.
BMP + Cl	6.3	n.d.	n.d.
PC + (PI, PS, PA)	34.0	20.3	8.9
SM + GM3	12.6	23.0	35.6
total	100	100	100

Li   om RN cells (Figure 3 lane 2), immuno-adsorbed exosomes (Figure 3, lane 3) and

membranes that did not bind to the immuno-beads (Figure 3, lane 4) were stained and

quantified by comparison with known amounts of reference lipids. The data are representative

for two independent experiments. Values shown are plus/minus 5 %. The numbers represent

relative amounts of lipid with respect to the total of identified lipids in the same membrane

preparation. Due to the low amount of material some lipids, especially those of the non-bound

material derived from unlabelled cells, could not be determined (n.d.). Cholesterol,

sphingomyelin (SM) and ganglioside GM3 (GM3) are relatively enriched in adsorbed

exosomes as compared to total cell membranes on the expense of the relative amount of

phosphatidyl ethanolamine (PE) and its respective ether lipids as well as phosphatidyl choline

(PC), phosphatidyl inositol (PI), phosphatidyl serine (PS) and phosphatidic acid (PA). Note

tha... P (LBPA) and the mitochondrial lipid cardiolipin (CL) comigrate and were not

detected in exosomes.

Table III A.

Mass spectrometric analyses of prominent RN-derived acidic lipids

Lipid	Fatty acid composition	[M-H]- at m/z	MSMS of [M-H]-
phosphatidyl inositol (PI)	C18:0, C20:3	887.5	283.3; 305.2
	C18:0, C20:4	885.5	283.3; 303.2
	C18:0, C18:1	863.5	283.3; 281.3
	C18:1, C18:1	861.5	281.3
	C18:0, C16:0	835.5	283.3; 255.3
phosphatidic acid (PA)	C18:0, C18:1	701.5	283.3; 281.3
phosphatidyl serine (PS)	C18:0, C18:1	788.5	283.3; 281.3
bismonoacyl glycerophosphate (BMP)	C18:0, C18:1	775.5	283.3; 281.3
	C18:1, C18:1	773.5	281.3
	C18:1, C16:0	747.5	281.3; 255.3
Cardiolipin (CL)	3 x C18:1; 1 x C16:1	713.5 ([M-2H]$^{2-}$)	281.3; 253.2
	2 x C18:1; 2 x C16:1	699.5 ([M-2H]$^{2-}$)	281.3; 253.2
ganglioside GM3	C24:1	1261.9	290.2
	C16:0	1151.8	290.2

Table III B. Mass spectrometric analyses of prominent RN-derived uncharged lipids

Lipid	Fatty acid composition	[M+Na]+ at m/z
phosphatidyl ethanolamine (PE)	C40:5	816.6
	C40:6	814.6
	C38:3	792.6
	C38:4	790.6
	C36:1	768.6
	C36:2	766.6
	C34:1	740.6
	C34:2	738.6
PE-ether	C38:5	774.6
	C38:6	772.6
	C34:1	726.6
	C34:2	724.6
phosphatidyl choline (PC)	C34:1	782.6; (760.6 [M+H]+)
	C36:4	782.6 [M+H]+
	C34:3	756.6 [M+H]+
	C32:0	734.6 [M+H]+
	C30:0	706.6 [M+H]+
sphingomyelin (SM)	C24:1	835.7
	C16:0	725.6
di...sphingomyelin	C16:0	727.6
cholesterol		369.4 [M+H-H2O]+

MSMS spectra were used to distinguish between proton or sodium adducts of the parent ions and to identify the lipid species by the relevant signals of their head group. Cholesterol ions were measured with the MALDI-TOF instrument in the positive ion mode.

LEGENDS OF THE FIGURES

FIGURE 1. Isolation of exosomes on sucrose density gradients

Exosomes were collected from RN culture media by differential centrifugation. Membranes that pelleted at 420,000 g$_{max}$ x min were floated up into a sucrose density gradient. Gradient fractions (from bottom to top, indicated 1 – 11) were diluted with PBS and membranes were collected by ultra-centrifugation and analysed by 12.5% SDS-PAGE and Coomassie blue staining (A) or Western blotting for MHC class II-β (B). Exosomes peaked in fractions 6-8. Arrows in A at the left indicate example proteins that did not co-migrate with exosomes; numbers at the right indicate molecular weight markers.

FIGURE 2. Immuno-purification of exosomes

Exosomes, isolated by differential centrifugation and floatation into sucrose gradients (as in Figure 1), were immuno-adsorbed to Dynal beads coated with anti-MHC class II or control IgG. The beads were collected and analysed by 7.5% (lanes 1-4) or 12.5% (lane 5) SDS-PAGE and Coomassie blue stained. Essentially all proteins were immuno-adsorbed to anti-MHC class II-coated beads (lanes 1 and 5) and little protein remained in the supernatant (lane 3). In contrast, essentially all protein remained in the supernatant (lane 4) of control IgG coated beads (lane 2), indicating the specificity of the procedure. Coomassie blue stained proteins were excised from lanes 1 and 5 and analysed by nano-electrospray tandem mass spectrometry. Identified proteins are indicated in the figure as well as in table I. mIgHC is immunoglobulin derived from the mouse anti-MHC class II antibody from the Dynal Beads.

FIGURE 3. Analysis of lipids from RN cells and exosomes

Lipids from total cellular membranes and Dynal bead-associated exosomes were extracted, subjected to thin-layer chromatography and stained. Lane 1, reference lipids, their nature is indicated on the left side of the figure: cholesterol (Chol); dioleoyl phosphatidyl ethanolamine (DOPE); cardiolipin (BHCL) from bovine heart; dioleoyl phosphatidyl choline (DOPC); sphingomyelin (SM) from bovine brain containing stearic or nervonic acid, resulting in a double band by TLC Lane 2, lipids from total RN membranes. Their nature is indicated at the right side of the figure and was determined in a parallel experiment in which metabolically ^{14}C-labelled lipids were extracted from cells, separated by TLC and analysed by mass spectrometry. Chol, Cholesterol; SM, sphingomyelin; GM3, ganglioside GM3; PE, phosphatidyl ethanolamine and its respective ether lipids; PC, phosphatidyl choline; PI, phosphatidyl inositol; PS, phosphatidyl serine; PA, phosphatidic acid, BMP, (Bismonoacyl glycerophosphate); CL, cardiolipin. Spot X is a non-identified compound; spot Y is not identified but has the mobility of monohexosylceramides; spot Z at the front could not be associated with any known lipid and is most likely related to compound(s) from plastics or other paraphernalia that were extracted by the solvents. Lane 3, lipids from Dynal bead-associated exosomes (isolated as in figure 2, lane 1). Lane 4, lipids from non-bound material (as figure 2, lane 3). The quantified relative amounts of lipids are depicted in table II.

FIGURE 4. Cholesterol, MHC class II and CD63 co-localize on exosomes

Cryo-sections of RN cells were double-labeled for cholesterol (10 nm gold) and MHC class II (15 nm gold) (A) or CD63 (15 nm gold) (B). Figure A shows a fusion profile between a MVB and the plasma membrane (PM) containing released exosomes (E). Arrows indicate the site of fusion. The plasma membrane of an opposing cell is indicated by PM*. Note the presence of MHC class II on the plasma membrane and on exosomes. Cholesterol is predominantly

31

present on exosomes. Figure B indicates a similar structure with exosomes (E) relatively enriched in both cholesterol and CD63 as compared to the plasma membrane (PM).

FIGURE 5. Detergent insolubility of exosomes

Samples of isolated exosomes were suspended in PBS containing either no detergent (contr, lane 1), CHAPS (lanes 2-7) or TX100 (lane 8). When indicated, the samples also contained EDTA (lane 3), MgCl$_2$ (Mg^{2+}, lane 4) or methyl-β-cyclodextrin (MβcD, lanes 6 and 7). The samples were incubated for 30 min at either 0°C (lanes 1-4, 6 and 8) or 37°C (lanes 5 and 7) and ultra-centrifuged at 4°C to sediment DRM. Pellets were analyzed either by SDS-PAGE and stained with Coomassie blue (A) or by Western blotting for MHC class II β (B). Closed arrows indicate example proteins that were entirely solubilised by detergents, open arrows indicate partially solubilised proteins, and arrow heads non-solubilised proteins. Molecular weight markers are on the right. The data are representative for three independent experiments.

FIGURE 6. Detergent-resistant exosomal proteins have a low buoyant density

Isolated exosomes were incubated in the absence (control) or presence of CHAPS and layered at the bottom of a sucrose density gradient. After ultra-centrifugation, gradient fractions were tested for the presence of MHC class II β-chain, CD81 and CD63 by Western blotting. Bottom fractions are at the left. About 1/3 of all three markers floated up into the gradient, indicating their association with DRM/rafts. The data are representative for three independent experiments.

32

FIGURE 7. Isolation of MIIC by density gradient electrophoresis

Intact RN cells were biotinylated to label plasma membrane proteins. The cells were then

homogenized and post-nuclear supernatants were fractionated by density gradient

electrophoresis. Anodal fractions are at the left. Fractions were analyzed for the presence of

total protein (A, closed circles), β-hexosaminidase (A, open circles), MHC class II β-chain

(B), and biotinylated plasma membrane proteins (C). MIIC localized to fraction 5-10, and

plasma membrane to fraction 14-21. The data are representative for three independent

experiments.

FIGURE 8. The solubility MHC class II depends on its location

In A, isolated exosomes and MIIC were incubated in the absence or presence of CHAPS or

TX100 at 0°C or 37°C as indicated. The samples were then ultra-centrifuged and the pellets

analyzed by Western blotting for MHC class II β. MHC class II from MIIC and exosomes has

similar solubilisation characteristics. In B, biotinylated intact cells and biotinylated exosomes

were incubated in the presence or absence of detergents as above. Biotinylated proteins were

extracted with Neutravidin beads from pelleted detergent resistant DRM that were

resuspended in TX100, and analyzed for the presence of MHC class II β-chain by Western

b   . Biotinylated MHC class II was almost entirely extracted by detergents from the

plasma membrane but not from exosomes or MIIC, indicating that the incorporation of MHC

class II in DRM depends on its subcellular location. The data are representative for three

independent experiments.

33

REFERENCE LIST

1. Piper, R. C. and Luzio, J. P. (2001) *Traffic.* 2, 612-621

2. Longva, K. E., Blystad, F. D., Stang, E., Larsen, A. M., Johannessen, L. E., and Madshus, I. H. (2002) *J.Cell Biol.* 156, 843-854

3. Kleijmeer, M., Ramm, G., Schuurhuis, D., Griffith, J., Rescigno, M., Ricciardi-Castagnoli, P., Rudensky, A. Y., Ossendorp, F., Melief, C. J., Stoorvogel, W., and Geuze, H. J. (2001) *J.Cell Biol.* 155, 53-63

4. Kleijmeer, M. J., Morkowski, S., Griffith, J. M., Rudensky, A. Y., and Geuze, H. J. (1997) *J.Cell Biol.* 139, 639-649

5. Pan, B. T., Teng, K., Wu, C., Adam, M., and Johnstone, R. M. (1985) *J.Cell Biol.* 101, 942-948

6. Stoorvogel, W., Kleijmeer, M. J., Geuze, H. J., and Raposo, G. (2002) *Traffic.* 3, 321-330

7. Denzer, K., Kleijmeer, M. J., Heijnen, H. F., Stoorvogel, W., and Geuze, H. J. (2000) *J.Cell Sci.* 113, 3365-3374

8. Peters, P. J., Geuze, H. J., Van der Donk, H. A., Slot, J. W., Griffith, J. M., Stam, N. J., Clevers, H. C., and Borst, J. (1989) *Eur.J.Immunol.* 19, 1469-1475

9. Raposo, G., Nijman, H. W., Stoorvogel, W., Liejendekker, R., Harding, C. V., Melief, C. J., and Geuze, H. J. (1996) *J.Exp.Med.* 183, 1161-1172

10. Escola, J. M., Kleijmeer, M. J., Stoorvogel, W., Griffith, J. M., Yoshie, O., and Geuze, H. J. (1998) *J.Biol.Chem.* 273, 20121-20127

11. Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J., Flament, C., Tenza, D., Ricciardi-Castagnoli, P., Raposo, G., and Amigorena, S. (1998) *Nat.Med.* 4, 594-600

12. Thery, C., Regnault, A., Garin, J., Wolfers, J., Zitvogel, L., Ricciardi-Castagnoli, P., Raposo, G., and Amigorena, S. (1999) *J.Cell Biol.* 147, 599-610

13. Thery, C., Boussac, M., Veron, P., Ricciardi-Castagnoli, P., Raposo, G., Garn, J., and Amigorena, S. (2001) *J.Immunol.* 166, 7309-7318

14. Raposo, G., Tenza, D., Mecheri, S., Peronet, R., Bonnerot, C., and Desaymard, C. (1997) *Mol.Biol.Cell* 8, 2631-2645

15. Heijnen, H. F., Schiel, A. E, Fijnheer, R., Geuze, H. J., and Sixma, J. J. (1999) *Blood* 94, 3791-3799

16. van Niel, G., Raposo, G., Candalh, C., Boussac, M., Hershberg, R., Cerf-Bersussan, N., and Heyman, M. (2001) *Gastroenterology* 121, 337-349

17. Skokos, D., Le Panse, S., Villa, I., Rousselle, J. C., Peronet, R., David, B., N mane, A., and Mecheri, S. (2001) *J.Immunol.* 166, 868-876

18. Denzer, K., van Eijk, M. Kleijmeer, M. J., Jakobson, E., de Groot, C., and Geuze, H. J. (2000) *J.Immunol.* 165, 1259-1265

19. Katzmann, D. J., Babst, M., and Emr, S. D. (2001) *Cell* 106, 145-155

20. Bishop, N., Horman, A., and Woodman, P. (2002) *J.Cell Biol.* 157, 91-101

34

21. Blanchard, N., Lankar, D., Faure, F., Regnault, A., Dumont, C., Raposo, G., and Hivroz, C. (2002) *J.Immunol.* **168**, 3235-3241

22. Raiborg, C., Bache, K. G., Gillooly, D. J., Madshus, I. H., Stang, E., and Stenmark, H. (2002) *Nat.Cell Biol.* **4**, 394-398

23. Reggiori, F. and Pelham, H. R. (2001) *EMBO J.* **20**, 5176-5186

24. Neefjes, J. J., Stollorz, V., Peters, P. J., Geuze, H. J., and Ploegh, H. L. (1990) *Cell* **61**, 171-183

25. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal. Chem.* **68**, 850-858

26. Mann, M. and Wilm, M. (1994) *anal.chem.* **66**, 4390-4399

27. Tulp, A., Verwoerd, D., Fernandez-Borja, M., Neefjes, J., and Hart, A. A. (1996) *Electrophoresis* **17**, 173-178

28. Koldovsky, O. and Palmieri, M. (1971) *Biochem.J.* **125**, 697-701

29. Iwamoto, M., Morita, I., Fukuda, M., Murota, S., Ando, S., and Ohno-Iwashita, Y. (1997) *Biochim.Biophys.Acta* **1327**, 222-230

30. Mobius, W., Ohno-Iwashita, Y., van Donselaar, E. G., Oorschot, V. M., Shimada, Y., Fujimoto, T., Heijnen, H. F., Geuze, H. J., and Slot, J. W. (2002) *J.Histochem.Cytochem.* **50**, 43-56

31. Ikonen, E. (2001) *Curr.Opin.Cell Biol.* **13**, 470-477

32. Brown, D. A. and London, E. (2000) *J.Biol.Chem.* **275**, 17221-17224

33. Tulp, A., Verwoerd, D., Dobberstein, B., Ploegh, H. L., and Pieters, J. (1994) *Nature* **369**, 120-126

34. Wolfers, J., Lozier, A., Raposo, G., Regnault, A., Thery, C., Masurier, C., Flament, C., Pouzieux, S., Faure, F., Tursz, T., Angevin, E., Amigorena, S., and Zitvogel, L. (2001) *Nat.Med.* **7**, 297-303

35. Hemler, M. E. (2001) *J.Cell Biol.* **155**, 1103-1107

36. Boucheix, C. and Rubinstein, E. (2001) *Cell Mol.Life Sci.* **58**, 1189-1205

37. Claas, C., Stipp, C. S., and Hemler, M. E. (2001) *J.Biol.Chem.* **276**, 7974-7984

38. Angelisova, P., Hilgert, I., and Horejsi, V. (1994) *Immunogenetics* **39**, 249-256

39. Serru, V., Le Naour, F., Billard, M., Azorsa, D. O., Lanza, F., Boucheix, C., and Rubinstein, E. (1999) *Biochem.J.* **340**, 103-111

40. Lagaudriere-Gesbert, C., Lebel-Binay, S., Wiertz, E., Ploegh, H. L., Fradelizi, D., and Conjeaud, H. (1997) *J.Immunol.* **158**, 2790-2797

41. Szollosi, J., Horejsi, V., Bene, L., Angelisova, P., and Damjanovich, S. (1996) *J.Immunol.* **157**, 2939-2946

42. Rubinstein, E., Le Naour, F., Lagaudriere-Gesbert, C., Billard, M., Conjeaud, H., and Boucheix, C. (1996) *Eur.J.Immunol.* **26**, 2657-2665

43. Anderson, H. A., Hiltbold, E. M., and Roche, P. A. (2000) *Nat.Immunol.* **1**, 156-162

44. Kropshofer, H., Spindeldreher, S., Rohn, T. A., Platania, N., Grygar, C., Daniel, N., Wolpl, A., Langen, H., Horejsi, V., and Vogt, A. B. (2002) *Nat.Immunol.* **3**, 61-68

45. Engring, A. and Pieters, J. (2001) *Int.Immunol.* **13**, 127-134

46. Hammond, C., Denzin, L. K., Pan, M., Griffith, J. M., Geuze, H. J., and Cresswell, P. (1998) *J.Immunol.* **161**, 3282-3291

47. Hutter, W. B. and Zimmerberg, J. (2001) *Curr.Opin.Cell Biol.* **13**, 478-484

48. Kobayashi, T., Beuchat, M. H., Chevallier, J., Makino, A., Mayran, N., Escola, J. M., Lebrand, C., Cosson, P., Kobayashi, T., and Gruenberg, J. (2002) *J.Biol.Chem.* **277**, 32157-32164

49. Fraile-Ramos, A., Pelchen-Matthews, A., Kledal, T. N., Browne, H., Schwartz, T. W., and Marsh, M. (2002) *Traffic.* **3**, 218-232

50. Raposo, G. and Marks, M. S. (2002) *Traffic.* **3**, 237-248

51. Garrus, J. E., von Schwedler, U. K., Pornillos, O. W., Morham, S. G., Zavitz, K. H., Wang, H. E., Wettstein, D. A., Stray, K. M., Cote, M., Rich, R. L., Myszka, D. G., and Sundquist, W. I. (2001) *Cell* **107**, 55-65

52. Aloia, R. C., Tian, H., and Jensen, F. C. (1993) *Proc.Natl.Acad.Sci.U.S.A* **90**, 5181-5185

53. Ono, A. and Freed, E. O. (2001) *Proc.Natl.Acad.Sci.U.S.A* **98**, 13925-13930

54. Johnstone, R. M., Adam, M., Hammond, J. R., Orr, L., and Turbide, C. (1987) *J.Biol.Chem.* **262**, 9412-9420

55. Rabesandratana, H., Toutant, J. P., Reggio, H., and Vidal, M. (1998) *Blood* **91**, 2573-2580

56. Moffett, S., Brown, D. A., and Linder, M. E. (2000) *J.Biol.Chem.* **275**, 2191-2198

57. Tsao, P., Cao, T., and von Zastrow, M. (2001) *Trends Pharmacol.Sci.* **22**, 91-96

58. Odorizzi, G., Babst, M., and Emr, S. D. (1998) *Cell* **95**, 847-858

59. Mathew, A., Bell, A., and Johnstone, R. M. (1995) *Biochem.J.* **308**, 823-830

60. Geminard, C., Nault, F., Johnstone, R. M., and Vidal, M. (2001) *J.Biol.Chem.* **276**, 9910-9916

61. Newmyer, S. L. and Schmid, S. L. (2001) *J.Cell Biol.* **152**, 607-620

62. Sachse, M., Urbe, S., Oorschot, V., Strous, G. J., and Klumperman, J. (2002) *Mol.Biol.Cell* **13**, 1313-1328

63. Ramm, G., Pond, L., Watts, C., and Stoorvogel, W. (2000) *J.Cell Sci.* **113**, 303-313

64. Agarraberes, F. A. and Dice, J. F. (2001) *J.Cell Sci.* **114**, 2491-2499

65. Defacque, H., Egeberg, M., Habermann, A., Diakonova, M., Roy, C., Mangeat, P., Voelter, W., Marriott, G., Pfannstiel, J., Faulstich, H., and Griffiths, G. (2002) *EMBO J.* **19**, 199-212

66. Sagara, J., Tsukita, S., Yonemura, S., Tsukita, S., and Kawai, A. (1995) *Virology* **206**, 485-494

67. Hecker, C., Weise, C., Schneider-Schaulies, J., Holmes, H. C., and ter, M., V (1997) *Virus Res.* **49**, 215-223

68. Ott, D. E., Coren, L. V., Kane, B. P., Busch, L. K., Johnson, D. G., Sowder, R C., Chertova, E. N., Arthur, L. O., and Henderson, L. E. (1996) *J.Virol.* **70**, 7734-7743

69. Babst, M., Katzmann, D., Estepa-Sabal, E., Meerloo, T., and Emr, S. (2002) *D v.Cell* **3**, 271

figure 1:

70. Babst, M., Katzmann, D., Snyder, W., Wendland, B., and Emr, S. (2002) *Dev.Cell* 3, 283

71. Bishop, N. and Woodman, P. (2001) *J.Biol.Chem.* 276, 11735-11742

72. Vidal, M., Mangeat, P., and Hoekstra, D. (1997) *J.Cell Sci.* 110, 1867-1877

73. Auger, I., Escola, J.M., Gorvel, J.P., and Roudier, J. (1996) *Nat.Med.* 2, 306-310

74. Lagaudriere-Gesbert, C., Newmyer, S. L., Gregers, T. F., Bakke, O., and Ploegh, H. L. (2002) *Proc.Natl.Acad.Sci.U.S.A* 99, 1515-1520

75. Penninger, J. M., Irie-Sasaki, J., Sasaki, T., and Oliveira-dos-Santos, A. J. (2001) *Nat.Immunol.* 2, 389-396

76. Van Dyke, R. W. (1996) *Subcell.Biochem.* 27:331-60, 331-360

77. Koopman, G., Keehnen, R. M., Lindhout, E., Newman, W., Shimizu, Y., van Seventer, G. A., de Groot, C., and Pals, S. T. (1994) *J.Immunol.* 152, 3760-3767

78. Gray, D., Kosco, M., and Stockinger, B. (1991) *Int.Immunol.* 3, 141-148
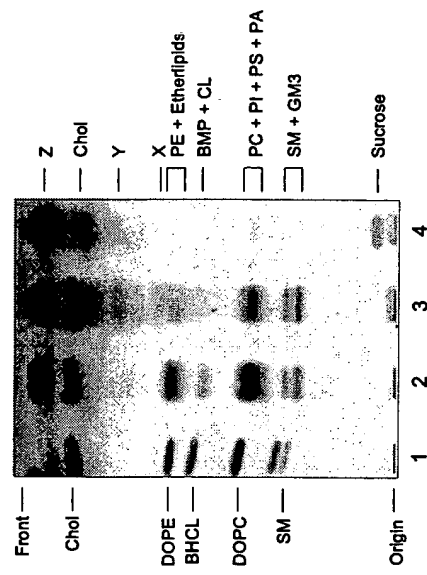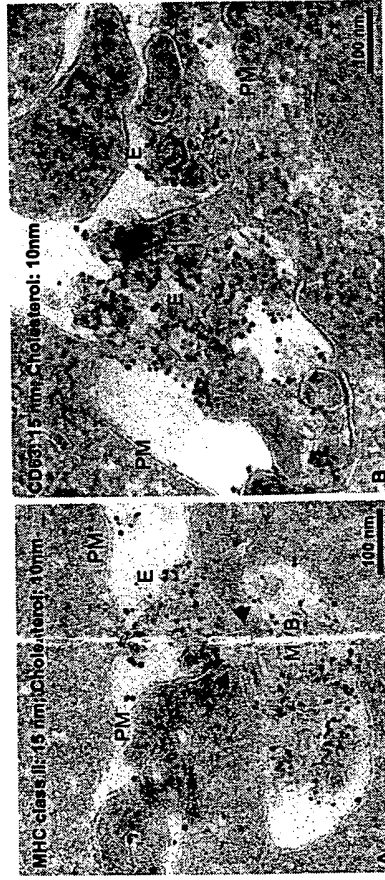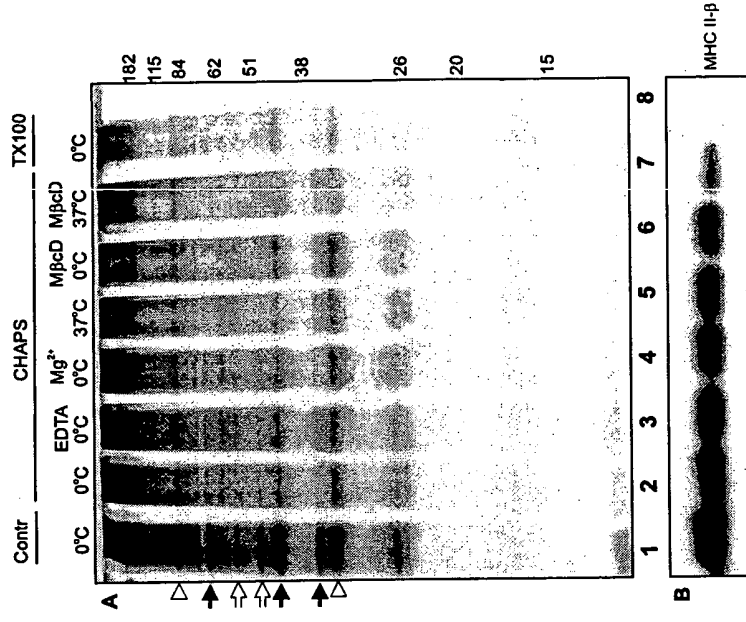
figure 3:

figure 2:

figure 5:

figure 4:

figure 7:

figure 6:

figure 8: